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p53 Pathway in Renal Cell Carcinoma Is Repressed by a Dominant Mechanism

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ABSTRACT

Renal cell carcinoma (RCC) rarely acquires mutations in *p53* tumor suppressor gene, suggesting that p53 signaling in this tumor type might be repressed by some other mechanism. In fact, all four RCC-derived cell lines we tested maintained wild-type *p53* but were not capable of transactivating p53-responsive reporters and endogenous p53-responsive genes. p53 protein in RCC showed normal response to genotoxic stress, including accumulation, nuclear translocation, and activation of specific DNA binding. Functional and expression analysis of Mdm2, MdmX, and Arf showed lack of involvement of these p53 regulators in the observed defect of p53 function in RCC. However, activation of p53-mediated transactivation could be achieved by extremely high levels of p53 attained by lentivirus vector-driven transduction, suggesting the involvement of a dominant inhibitor in repression of p53-dependent transactivation in RCC. Consistently, p53 inactivation prevailed in the hybrids of RCC cells with the cells possessing fully functional p53. Remarkably, cells of normal kidney epithelium also caused partial p53 repression in cell fusion experiments, suggesting that RCC-specific p53 repression may be based on an unknown dominant mechanism also acting in normal kidney tissue.

INTRODUCTION

Renal cell carcinoma (RCC) accounts for approximately 85% of all kidney cancers. Approximately 30,000 patients in the United States were diagnosed with renal cancers in 1999, with an estimated 17,800 deaths (1, 2). This tumor is characterized by exceptionally high resistance to radiation and chemotherapy (3), which can be explained in part by naturally high levels of expression of multidrug transporter and elevated activity of the glutathione system in RCC progenitors, kidney tubular epithelial cells. However, treatment resistance of tumor cells can also be associated with the suppression of apoptotic mechanisms of cellular response to stresses, such as the p53 tumor suppressor pathway (4). In fact, in many other tumor types, mutations in *p53* are associated with poor treatment outcome (5). This is not the case for RCC, whose high intrinsic resistance to treatments is accompanied by a very low frequency of mutations in *p53*, as follows from the analysis of several *p53* mutation databases (6, 7) and immunohistochemical staining of RCC tissue blocks for p53, presuming that mutations in *p53* result in the accumulation of the protein (8–12).

Although *p53* remains wild type in the majority of RCCs, this does not mean, as we know from other tumor types, that it is functional. The p53 function can be repressed by some other mechanisms, which may involve overexpression of natural negative regulators of p53, such as Mdm2 or MdmX (13–15), or loss of positive regulators, such as Arf (16, 17), or by viral proteins, such as E6 of the human papilloma virus (18). Hence, the p53 pathway could be nonfunctional in RCC as well, regardless of the lack of mutations in the p53 gene itself. Determination of the status of p53 function in RCC and, if the function is repressed, identification of the mechanism for this repression should be important for understanding the genetic basis underlying the unique properties of RCC and for developing specific therapeutic approaches. The present study is the first step of this program. We found that p53 signaling is indeed repressed in RCC cell lines in an unusual way: the wild-type p53 in these cells undergoes all essential steps of activation conversions in response to DNA damage, including stabilization, nuclear accumulation, and DNA binding, but fails to activate transcription. The cell-cell fusion experiments indicate that this repression is determined by a dominant mechanism that seems to be a characteristic of normal RCC progenitors, cells of normal kidney epithelium. None of the most common p53-regulatory factors was found responsible for the repression, suggesting that RCC uses a unique tissue-specific mechanism of p53 repression.

MATERIALS AND METHODS

Cell Lines. Primary RCC cell lines (RCC 26b, RCC 45, RCC 54, and RCC 72) were provided by James Finke (The Cleveland Clinic Foundation); normal kidney tubular epithelia were kindly provided by Joseph Di Donato (The Cleveland Clinic Foundation). Other cell lines used [ACHN (RCC), MCF7 (breast cancer), HT1080 (lung fibrosarcoma), H1299 (lung adenocarcinoma), HeLa (cervical carcinoma), A293 (embryonic kidney epithelial cells transformed with DNA of adenovirus type 5), and LNCaP (prostate adenocarcinoma)] were purchased from American Type Culture Collection. All cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 10 mM HEPES buffer, 55 nM β-mercaptoethanol, and antibiotics.

Plasmids. Plasmids encoding wild-type p53, dominant-negative p53 element GSE56, and Arf cDNA were generated by insertion of p53 cDNA, GSE56 DNA, or Arf cDNA into retroviral vector pLXSN (Clontech) or its derivative pLXSP, obtained by substitution of neo selectable marker to puromycin resistance. pBabe-siHdm2 plasmid was generated by insertion of H1 promoter into the right long terminal repeat of pBabe-puro retroviral vector followed by the template for small interfering RNA (siRNA) expression, designed according to the loop model described by Brummelkamp *et al.* (19). The following oligonucleotide was chosen from the *Hdm2* sequence: CTTCGGAACAAGAGACCCT. p21-ConA-Luc plasmid contains luciferase cDNA under the control of a cassette of three different p53-binding elements [high-affinity 20-bp p53-binding site Con (20), 50-bp p53-binding site fragment A from ribosome gene cluster (21), and six copies of 20-bp p53-binding site from human p21Waf1/Cip1 gene (22) and the minimal Hsp70 promoter]. pHsp70Luc plasmid contains only Hsp70 promoter. pUST-p21-ConA-LacZ (pConA-LacZ) is a self-inactivated retroviral vector with the same reporter elements as described for pConALuc plasmid, except that minimal Hsp70 promoter is substituted with minimal cytomegalovirus (CMV) promoter. pGL3-Bax-Luc reporter (pBax-Luc) was obtained from Moshe Oren (Weizmann Institute of Science) and contains luciferase under the control of a 370-bp element of the *Bax* promoter including the TATA box. pCMVLacZ plasmid encodes bacterial β-galactosidase under the control of CMV promoter (Promega).

Chemicals. 5-Fluorouracil, doxorubicin, camptothecin, Taxol, and polyethylene glycol (FW 1300–1500) were purchased from Sigma.

Sequencing of p53 cDNA. Sequencing of *p53* was done using a previously described protocol (23). Briefly, full-length *p53* cDNA was generated by reverse transcription-PCR reaction of total RNA isolated from RCC cell lines, using oligo(dT) as reverse transcription primer and p53-specific primers for PCR. Then the products of the PCR reaction were sequenced using an automated sequencing protocol by The Cleveland Clinic Foundation DNA sequencing core facility. All sequences obtained were compared with wild-type *p53* sequence contained in GenBank (GenBank accession number AF307851).

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Retroviral and Lentiviral Transduction. Packaging cells (A293 from Clontech) plated in 60-mm plates were transfected with 2 μg of retroviral vector DNA using LipofectAMINE Plus (Invitrogen) according to the manufacturer's recommendations. The medium was changed after 8 h. Virus-containing media supplied with 8 μg/ml Polybrene (Sigma) were collected at 24 and 48 h after transfection and used for infection. Virus-transduced cells were selected for resistance to an appropriate selective agent (G418, hygromycin, or puromycin, depending on the vector) up to a complete death of noninfected cells.

Full-length cDNA for human *p53* was cloned into lentiviral vector pLV-CMV-H4 (kindly provided by Inder Verma; Salk Institute) expressing insert from the CMV promoter and puromycin resistance gene from histone H4 promoter. Stocks of recombinant lentiviruses carrying p53 or enhanced green fluorescent protein (control vector) were prepared using the 293T cell line transfected with pLV-CMV-p53 and pLV-CMV-EGFP plasmids along with packaging plasmids encoding viral structural proteins and G-protein of vesicular stomatitis virus using LipofectAMINE reagent (Invitrogen). Virus-containing media from 293T cells were collected 48 h later and transferred to target cells in the presence of 4 μg/ml Polybrene, and the virus was concentrated 50–100 times by ultracentrifugation. Virus titers (typically 10^8 IU/ml) were determined by infection of Rat1a cells (which are known to be resistant to ectopic expression of p53), followed by selection on puromycin and counting colonies.

Semiquantitative Reverse Transcription-PCR and Northern Blot Analyses. RNA was isolated from cell lines using Trizol reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Single-stranded cDNAs were synthesized using SuperScript II reverse transcriptase (Life Technologies, Inc.) and random hexamers as primers. The cDNAs were amplified using Advantage polymerase mix (Clontech) in a Peltier thermocycler (DYAD) for 28–35 cycles (depending on the target), with the exception of glyceraldehyde-3-phosphate dehydrogenase (23 cycles). Each cycle consisted of a 1-min denaturation at 95°C, a 45-s annealing at 56°C, and a 1-min extension at 68°C. Primers for PCR were as follows: p53s, 5′-GCCCCTCCTCAGCATCTTATCCG; p53as, 5′-TCCCAGGACAGGCACAAACACGC; Hdm2s, 5′-ACCAGGAGTCCTACCCTCTGTCAGTGTC; Hdm2as, 5′-GATAGACGTAATCCCAAAGCAGTCTACAGTC; Hdm4s, 5′-CAATCAGGTACGACCAAAACTGCC; and Hdm4as, 5′-GGGTTCTTTACGGAGAAGCTCTGACG.

The PCR detection of ARF and p16 RNAs was done as described in Ref. 16.

For Northern analysis, 10 μg of total RNA in glyoxal buffer, denatured for 15 min at 70°C, were loaded into the wells of precast Reliant gels (BMA, Rockland, ME) and run according to the manufacturer's instructions. After transferring RNA onto a Hybond-NX membrane (Amersham), blots were hybridized with specific probes according to the ExpressHyb protocol (Clontech). Radionucleotide-labeled probes were synthesized using the MegaPrime labeling kit (Amersham) according to the manufacturer's instructions with the following templates: full-length p53 cDNA used for sequencing and fragments of Hdm2, human ARF, and human glyceraldehyde-3-phosphate dehydrogenase cDNA provided by Clontech.

Western Blot Analysis. Cells were lysed in radioimmunoprecipitation assay buffer [25 mM Tris-HCl (pH 7.2), 125 mM NaCl, 1% NP40, 1% sodium deoxycholate, and 1 mM EDTA] containing 1 mM phenylmethylsulfonyl fluoride (Sigma), 10 μg/ml aprotinin (Sigma), and 10 μg/ml leupeptin (Sigma). Protein concentrations were determined with the Bio-Rad Dc protein assay kit. Equal protein amounts were run on gradient 4–20% precast gels (Novex) and blotted onto polyvinylidene difluoride membranes (Amersham). The following antibodies were used: anti-p53, monoclonal mouse DO1 (Santa Cruz Biotechnology); anti-p21, monoclonal mouse F-5 (Santa Cruz Biotechnology); and anti-mdm2, monoclonal mouse SMP14 (Santa Cruz Biotechnology). p53 phosphorylation status was analyzed using a phospho-p53 sampler kit from Cell Signaling according to the manufacturer's recommendations. Horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. Quantitation of the data was performed using Quantity One software from Bio-Rad.

p53 Transactivation Reporter Assay. Cells (2×10^5) were plated into 6-well plates and, after overnight incubation, transfected with LipofectAMINE Plus reagent (Life Technologies, Inc.) with 0.5 μg of p21-ConA-Luc, pBax-Luc or pHsp70-Luc plasmid in combination with different concentrations of pLp53SP, pLGSE56SP, pcDNA3wtp53, or pcDNA3ARF. pLXSP or pcDNA3 empty vectors were added into all transfections up to 2 μg of total DNA amount. Normalization of transfection efficiency was done by adding 0.2 μg of pCMV-LacZ plasmid. Luciferase activity and β-galactosidase activity were measured in lysates prepared 48 h after transfection with Cell Lysis Buffer (Promega) by luciferase assay system (Promega) or β-galactosidase enzyme system (Promega). Luminometric and colorimetric reactions were read on the Wallack 1420 plate reader (Perkin-Elmer).

Electrophoretic Mobility Shift Analysis. Nuclear extracts were prepared as described previously (24). Annealed oligonucleotide corresponding to p53 consensus element, PG5 (24), was radiolabeled with [α-^{32}P]dCTP by Klenow polymerase and then with [γ-^{32}P]dATP by T4 polynucleotide kinase. Labeled oligonucleotide (10^7 cpm) was affinity purified on Probe Quant columns (Amersham). Radiolabeled oligonucleotide was added to 20 μg of protein nuclear extract together with 1 μg of poly(deoxyinosinic-deoxycytidylic acid) (Amersham) to prevent nonspecific binding and incubated for 30 min at room temperature. For supershift, 200 ng of anti-p53 antibodies PAb421 were added to the reaction. After incubation, the entire reaction mixtures were loaded into 4% polyacrylamide gel in 0.5× Tris-borate EDTA buffer and run for 2 h at 200 V. Dried gels were exposed to X-ray films for 30 min to 1 h.

Cell Fusion Experiments. Two types of cells (5×10^6 of each type) was mixed together and plated onto a 100-mm tissue plate in regular medium. After a 3-h incubation, cells were washed three times with PBS solution, and 1 ml of reconstituted polyethylene glycol (Sigma) was added to the plate for 1 min. After this treatment, the cell layers were carefully washed three times with PBS and once with serum-free medium. Then regular medium was added. Twenty-four h later the cells were washed with PBS, fixed with 2% formaldehyde/0.5% glutaraldehyde solution in PBS for 15 min, and then stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as described previously (25). After overnight incubation at 37°C, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside solution was removed and replaced with DAPI solution (1 μg/ml in H_2O; Sigma) for 10 min to ensure heterokaryon formation.

RESULTS

RCC Cells Express Wild-Type p53 That Is Deficient in Transactivation. Full-length *p53* cDNA was amplified by PCR from total RNA of four RCC cell strains and one RCC cell line (ACHN). Sequence analysis showed that all cell variants analyzed expressed mRNA for wild-type p53. This finding is consistent with literature data indicating that the *p53* gene is infrequently altered in RCC (6–12).

To test the functionality of p53 protein in RCC cell lines, we estimated the ability of p53 to transactivate exogenous and endogenous p53-responsive promoters. Two types of reporter constructs were used in the transient transfection assay: p21-ConA-Luc and pBax-Luc. The p21-ConA cassette consists of p53-responsive element(s) representing the high-affinity consensus binding site Con (20), 50-bp fragment A from a ribosome gene cluster (21), and six distal p53-binding sites from the promoter of the p21/Waf1 gene (22) and minimal promoter of the Hsp70 gene (Fig. 1*A*). p53 specificity of the p21-pConA-Luc reporter is demonstrated by the results in Fig. 1*C*. The reporter showed no activity in p53-deficient H1299 cells, but was active in p53 wild-type HT1080 cells. Reporter activity in both cell types was induced in a dose-dependent manner by cotransfection of the plasmid expressing wild-type p53. In HT1080 cells, reporter activity was inhibited dose dependently by GSE56, a strong dominant negative p53 mutant (Ref. 26; Fig. 1*C*). Human kidney cell line A 293 provides an example of cells in which the activity of wild-type p53 is impaired by interacting with adenoviral protein E1b. Consistently, we observed almost no induction in reporter activity in response to exogenous p53, but GSE56 did suppress basal level(s) of reporter activity.

We compared transactivation activity of endogenous p53 in RCC and non-RCC cells using transient transfection of the p21-ConA-Luc and Bax-Luc reporter constructs. Activity of both reporters was extremely low in RCC, compared with non-RCC tumor cells

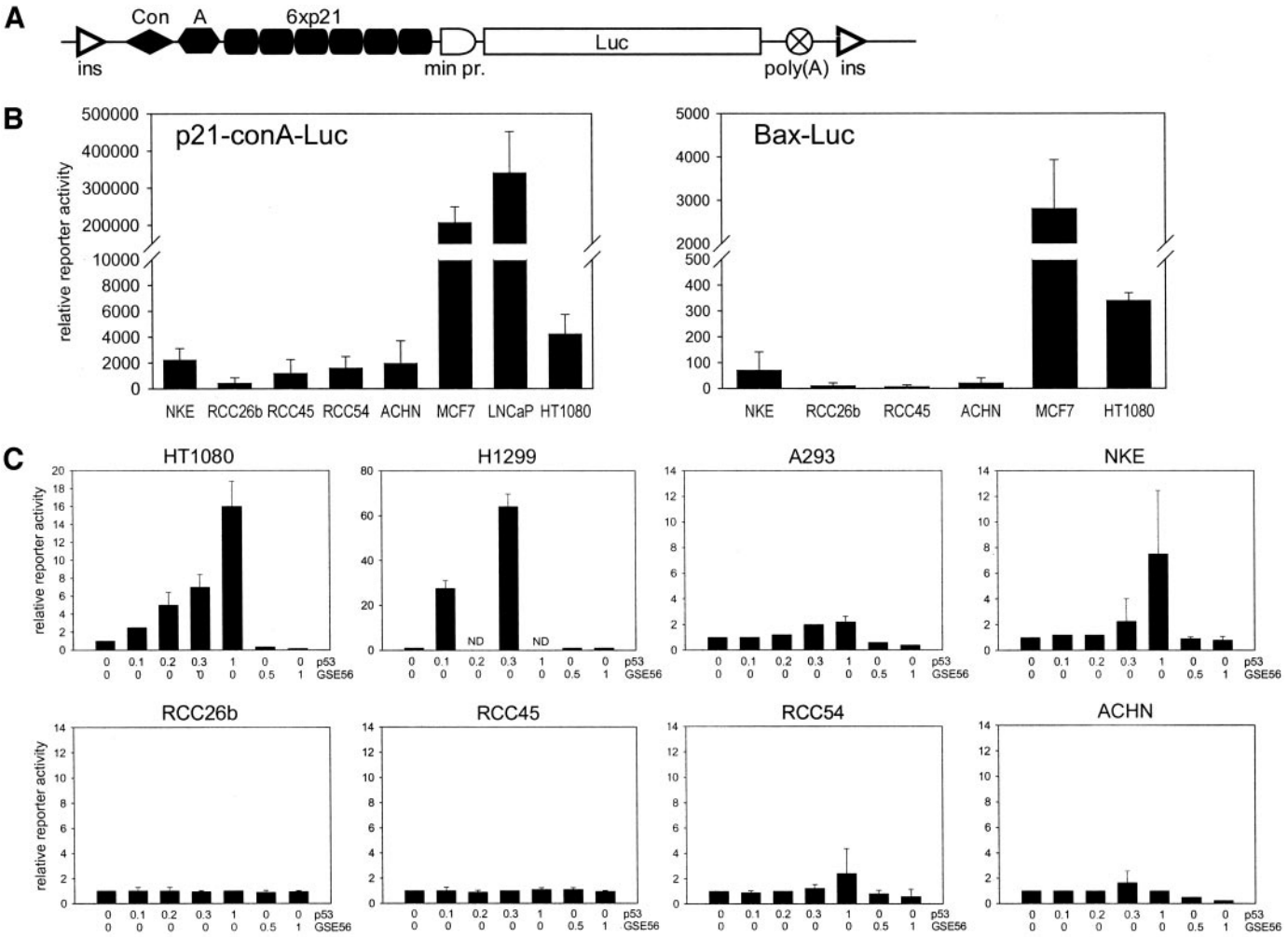

Fig. 1. Activity of p53-responsive reporters in different cell lines. *A,* scheme of p21-ConA-Luc vector (see the explanation in "Materials and Methods"). *ins,* insulator sequences; *Luc,* luciferase gene; *poly(A),* polyadenylation signal; *p21, Con,* and *A,* three different p53-binding sites. *B,* basal activity of p21-ConA-Luc and Bax-Luc reporters. Cells in 6-well plates were transfected with either of the reporters (0.5 μg/well p21-ConA-Luc; 2 μg/well Bax-Luc); PCMV-LacZ vector (0.1 μg/well) was added in all mixtures to estimate transfection efficiency. Plotted values represent luciferase activity, normalized by the transfection efficiency. *C,* influence of ectopic expression of p53 or GSE56 on p21-ConA-Luc reporter activity. The cells were transfected with p21-ConA-Luc plasmid (0.5 μg/well) in combination with the indicated amounts of pLp53SP or pLGSE56SP vectors (pLXSP empty vector was added where required to equilibrate DNA content). Data represent the fold induction of luciferase activity, normalized by transfection efficiency to basal level of luciferase activity (in case of transfection of only pConALuc vector).

(MCF7, HT1080, and LNCaP; Fig. 1*B*). Interestingly, in NKE cells, basal activity of the reporters was also rather low, but it was higher than that in the RCC-derived cell lines (Fig. 1*B*). Furthermore, ectopic expression of p53 in RCC cells (transient transfection of p53-expressing construct) did not cause reporter activation, whereas in other tumor cells, as well as in NKE cells, it induced a dose-dependent response of luciferase expression (Fig. 1*C*). In normal kidney epithelial cells, the response to exogenous p53 was attenuated, compared with HT1080 or H1299 cells (Fig. 1*C*). Expression of the dominant negative p53 mutant GSE56 inhibited basal levels of luciferase expression in NKE, ACHN, and, to a lesser degree, RCC54 cells (Fig. 1*C*).

Application of additional DNA-damaging treatments (UV, camptothecin, or doxorubicin) did not increase p21-ConA-Luc reporter activity in RCC cells (Fig. 2*A*). Hence, RCC cells were incapable of transactivating p53-responsive reporter constructs by both endogenous and ectopically expressed p53. Similar results were obtained when we used stable transduction of studied cells with reporter constructs instead of transient transfection. Three of the RCC cell lines (RCC26b, RCC45, and ACHN), as well as MCF7 cells, were transduced with self-inactivating retroviral vector containing the same p21-ConA promoter cassette (with minimal Hsp70 promoter substituted with minimal CMV promoter) that drives expression of another reporter, β-galactosidase. After elimination of nontransduced cells by puromycin selection (the puromycin resistance gene is driven by enhancer-less histone H4 promoter in the retroviral vector), the cells were treated with different concentrations of DNA-damaging drugs (Fig. 2*B*). Strong response of the reporter was observed in MCF7 cells, whereas RCC45 showed marginal reporter activation, and RCC26b and ACHN cells did not respond at all.

We further tested whether transactivation of endogenous targets by p53 in RCC cells would be as repressed as that of artificial reporter constructs. Specifically, we looked at the levels of p21Waf1 protein encoded by p53-inducible gene in response to high levels of ectopically expressed p53. Because p21Waf1 promoter could be activated by DNA damage in a p53-independent manner (27), we used lentivirus vector transduction of wild-type p53 instead of transient transfection. Lentivirus-driven p53 was effective in p21 induction in all cell lines tested except RCC (Fig. 3), thus providing additional confirmation that RCC cells are deficient in p53-dependent transactivation.

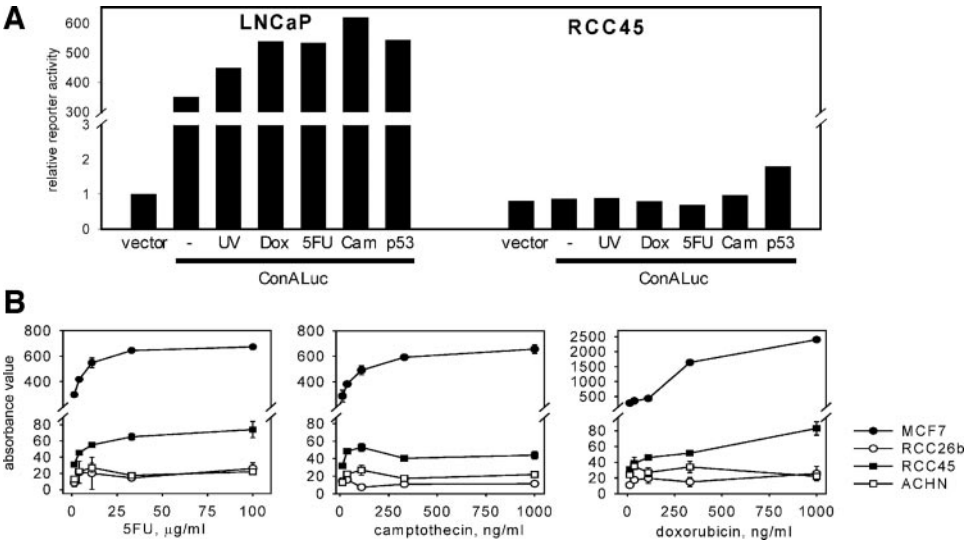

Fig. 2. Induction of p53-responsive reporter activity by DNA damage. *A*, induction of p21-ConA-Luc reporter activity by DNA damage in transient transfection assay. Cells were transfected with pConALuc plasmid (0.5 μg/well) alone or in combination with pLp53SP (0.5 μg/well). Twenty-four h after transfection, DNA damage was induced in the cells, where indicated, in the form of UV, doxorubicin (200 ng/ml), 5-fluorouracil (50 μg/ml), or camptothecin (150 ng/ml). Data represent luciferase activity, normalized by transfection efficiency. *B*, induction of integrated p21-ConA-LacZ reporter activity by DNA damage. Cells were transduced with retroviral vector containing β-galactosidase, regulated by p21-ConA promoter. After selection of puromycin-resistant clones, pooled populations were plated in 96-well plates and treated with the indicated reagent for 16 h. β-Galactosidase activity was estimated in cell lysates by colorimetric assay. Data represent β-galactosidase activity normalized by protein content in cell lysates.

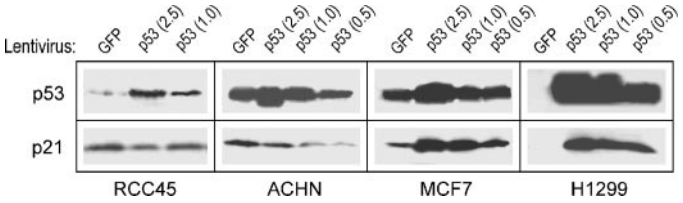

Fig. 3. Influence of increased expression of p53 on the level of p21/Waf1. Cells in 6-well plates were transduced with either green fluorescent protein or p53 lentiviral stocks (indicated as transduction units per ml $\times$ 10^7). Seventy-two h later, cell lysates were prepared and used for gel electrophoresis and Western blotting with subsequent immunodetection using anti-p53 (DO1) or anti-p21/Waf1 (F-5) antibodies.

p53 Protein in RCC Cells Responds to DNA Damage by Nuclear Accumulation and Specific DNA Binding. To approach the mechanisms of impaired transactivation of p53 in RCC, we analyzed p53 protein response to DNA damage. Specifically, we looked at p53 phosphorylation, accumulation, nuclear translocation, and specific DNA binding: the events that normally follow DNA damage in cells with a functional p53 pathway, but which can be deregulated in tumor cells (28).

We compared p53 phosphorylation by Western blot analysis with antibodies specific to different p53 phosphorylated sites in response to DNA-damaging stress (doxorubicin treatment) in RCC45 and MCF7 cells (Fig. 4*A*). Surprisingly, the majority of sites, including Ser^{6}, Ser^{15}, Ser^{46}, and Ser^{392}, were phosphorylated even more strongly in RCC45 cells than in MCF7 cells. We concluded that generally, phosphorylation of p53 at major sites is not affected in RCC. Consistent with these findings, we observed that p53 is stabilized in response to different types of DNA-damaging stress (UV radiation; Fig. 4*C*).

Immunofluorescence analysis of p53 expression and localization showed nuclear accumulation of p53 protein after DNA damage in all RCC cell variants tested (Fig. 4*C*). No obvious differences were found among RCC, normal kidney epithelium, MCF7, and HT1080 cells. These observations excluded cytoplasmic sequestration and lack of accumulation as potential mechanisms of functional repression of p53 in RCC cell lines.

DNA binding activity of p53 in RCC was estimated by a gel shift assay, using double-stranded oligonucleotide corresponding to p53 consensus DNA-binding element (Ref. 29; Fig. 5). Nuclear extracts were prepared from UV-treated (Fig. 5*A*) and untreated cells as well as cells treated with several other DNA-damaging stimuli (Fig. 5*B*). For comparison, we used nuclear extracts from normal kidney epithelia, MCF7, and HT1080 cells (all carrying wild-type *p53*) and H1299 cells (*p53* null). This assay also showed no differences between p53 from RCC and cell lines with an active p53 pathway: DNA-damaging treatment induced strong DNA binding in both groups of cell lines (Fig. 5). Hence, specific DNA binding activity of p53 protein seems to be appropriately induced in RCC cells by DNA

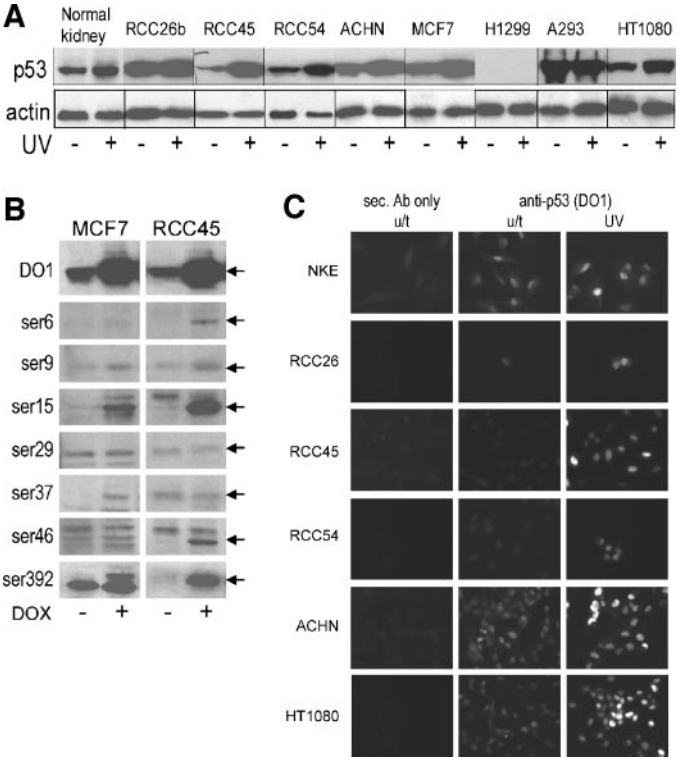

Fig. 4. Response of p53 protein to DNA damage in renal cell carcinoma (RCC) cells. *A*, levels of nuclear p53 protein are increased in different RCC cells after UV treatment. Results of Western blot analysis of lysates of indicated cells, either untreated or 16 h after treatment with UV, using DO1 antibody. *B*, comparison of phosphorylation of p53 in MCF7 and RCC cells 16 h after treatment with 400 ng/ml doxorubicin (*DOX*). Results of Western blot analysis of specific phosphorylated forms of p53 using antibodies against the indicated sites of phosphorylation. *C*, cells were irradiated by UV and fixed 16 h after treatment. Results of immunofluorescent detection of p53 in UV-treated (*UV*) and untreated (*u/t*) cells are shown. The *left column* shows cells stained with secondary antibodies only (control of specificity).

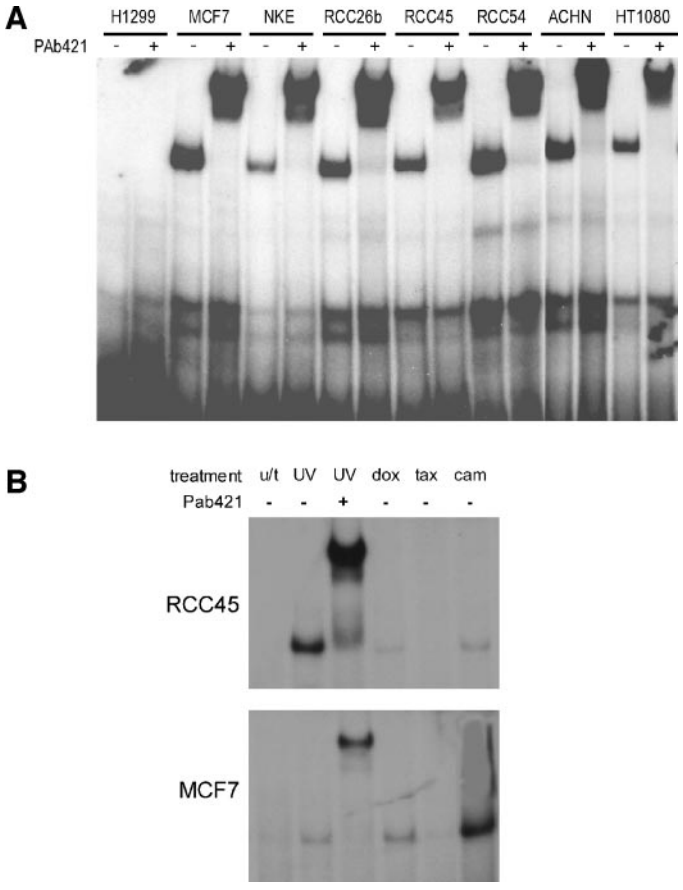

Fig. 5. DNA binding activity of p53 in different cell lines determined by electromobility shift assay with oligonucleotide corresponding to the consensus p53-binding element. Nuclear extracts (normalized by protein content; 10 μg) were incubated with p53-specific radioisotope-labeled double-stranded oligonucleotide PG5 (see "Materials and Methods"), with or without p53-specific antibodies PAb421 (as indicated). After incubation, the whole reaction mixtures were loaded into 4% polyacrylamide gel and run for 2 h at 100 V, after which the gel was dried in a vacuum drier and exposed to X-ray film for 1–2 h. *A*, indicated cells were treated with doses of UV causing maximal accumulation of p53 protein; lysates were prepared overnight after treatment. *B*, the indicated cells were either untreated (*u/t*) or treated with UV or several chemotherapeutic drugs [*dox*, doxorubicin (400 ng/ml); *tax*, Taxol (100 ng/ml); *cam*, captothecin (200 ng/ml)]. Lysates were prepared 8 hours after treatment.

damage, suggesting that factors blocking p53-dependent transactivation act further downstream in the pathway.

No Indications of Mdm2, MdmX, or Arf Involvement in Functional Repression of p53 in RCC. Deregulation of p53 pathway members can functionally block the activity of p53 signaling in some tumors (13–17). This mechanism commonly involves Mdm2 (known as Hdm2 in humans), MdmX (Hdm4 in humans), and Arf proteins acting as regulators of p53 protein stability and localization. Mdm2, encoded by an oncogene that is frequently overexpressed and amplified in tumors, is a ubiquitin ligase that physically binds p53 and promotes its ubiquitination and proteasomal degradation (13). Arf, which is encoded by a tumor suppressor gene transcribed from the same *Ink4a* locus as p16, is a natural inhibitor of Mdm2-p53 interaction that acts as a p53 cooperator (30). Its frequent loss in tumors is usually accompanied by reduced levels of p53 protein due to increased Mdm2-mediated degradation (17). MdmX is another p53 inhibitor, acting through p53 binding without causing p53 degradation, that can also be up-regulated in tumors (15).

Levels of mRNA expression of all these factors and the p53 gene itself were analyzed by semiquantitative reverse transcription-PCR and Northern blot hybridization (Fig. 6). No overexpression of Mdm2 or MdmX was observed in RCC cell lines as compared with normal kidney epithelia and the other tumor cell lines used (Fig. 6, *A*, *B*, and *D*). There was even a slight decrease in the amount of Mdm2 transcript in kidney cancer cells (Fig. 6*D*), which might be indicative of impaired p53 transactivation function in RCC because Mdm2 is a target of p53-dependent transactivation. We also did not find any differences in Hdm2 protein level in RCC and NKE cells (Fig. 6*B*).

Analysis of expression of p14Arf was done by reverse transcription-PCR in parallel with p16Ink4a because these tumor suppressor genes are encoded by the same locus and are frequently inactivated in combination by DNA methylation or deletions (30). Loss of Arf expression was previously demonstrated to be a frequent event in RCC (31). Expression of p14ARF and p16INKa mRNAs was assayed simultaneously using a combination of three primers with antisense corresponding to exon 2, shared between two mRNAs, and two sense primers, specific for the gene-specific exons (16). The data obtained showed the presence of both transcripts in normal kidney cells and loss of expression in all RCC cell lines except RCC26, in which expression of p14Arf but not p16Ink4a mRNA was preserved (Fig. 6*C*). Consistent with published data, HT1080 cells expressed neither of the two tested genes, whereas MCF7 cells showed marginal expression of Arf. Absence of Arf transcript in RCC was confirmed by Northern blot hybridization (Fig. 6*A*).

To verify whether the deficiency in Arf expression could contribute to p53 functional repression in RCC, we tested p53 transactivation function in RCC cells after reconstitution of Arf expression by transient transfection. Plasmid expressing human Arf cDNA from CMV promoter was cotransfected into a panel of cell lines with p21-ConA-Luc reporter with and without p53-expressing plasmid. As clear from the results presented in Fig. 7*A*, Arf expression had no effect on the activation of reporter construct in RCC, as opposed to NKE, HCT116 (either wild-type or p53-null variants), and U2-OS cells (Fig. 7*B*), all of which demonstrated strong dependence of p53-mediated transactivation of the reporter on Arf expression.

There is a formal possibility that Mdm2 inhibits p53 function through a mechanism that does not involve its overexpression. To test

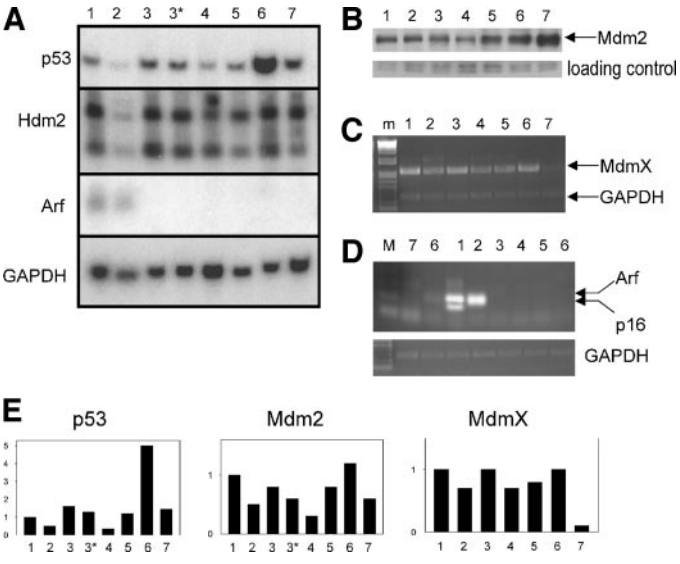

Fig. 6. Expression of p53 and p53 pathway members in normal kidney epithelial cells (*Lane 1*), RCC cell lines [RCC26b (*Lane 2*), RCC45 (*Lane 3*), RCC54 (*Lane 4*), ACHN (*Lane 5*) and MCF7 (*Lane 6*) and HT1080 (*Lane 7*) cells. *A*, Northern blot analysis of *p53*, *Mdm2*, and *ARF* expression (*, RCC45 cell RNA was loaded two times). *B*, Western blot analysis of Mdm2 expression using SMP14 monoclonal antibody (Santa Cruz Biotechnology). *C*, reverse transcription-PCR analysis of *MdmX* expression. *D*, reverse transcription-PCR analysis of *ARF* and *p16INKa* expression. *E*, quantitation of the data presented in *A*–*C*, normalized by glyceraldehyde-3-phosphate dehydrogenase expression using Quantity One software (Bio-Rad).

this hypothesis, we knocked down Mdm2 gene expression in RCC cell lines (shown for ACHN cells) by using retroviral construct pBabe-puro-H1siRNA expressing siRNA against this gene (Fig. 7*B*); human osteosarcoma U2-OS cells, in which p53 activity is repressed by overexpression of Hdm2, and vector expressing siRNA against EGFP were used as controls. Both lines carried integrated p21ConA-Luc reporter construct. After selection of transduced cells for puromycin resistance, reporter activity was measured in all cell variants. Whereas in U2-OS cells knockdown of Hdm2 resulted in increased activity of the reporter, no activation of luciferase was found in RCC cell lines (shown for ACHN cells in Fig. 7*B*). These results allow us to exclude a role for Mdm2 in p53 repression in RCC.

p53 Suppression in RCC Is Dominant in Cell-Cell Hybrids and Can Be Overcome by High Levels of Ectopically Expressed p53. There are two principle mechanisms that could be responsible for p53 inhibition in RCC cells: (*a*) p53 activity may be repressed by an inhibitory factor (dominant mechanism); or (*b*) it might result from the loss of an essential component of p53 signaling (recessive mechanism). To distinguish between these mechanisms, we used two approaches. First, we tested whether the block of p53-dependent transactivation could be overcome by increasingly high levels of ectopically expressed p53 reached with the help of lentivirus transduction. We used a broad range of concentrations of p53-transducing lentivirus to infect cells of RCC (RCC45) and non-RCC (MCF7) origin, both carrying p21-ConA-LacZ reporter (Fig. 8). We found that the expression of p53-responsive construct became induced in RCC45 cells that received the highest virus titers; expression of the same reporter in MCF7 cells reached a plateau at a much lower multiplicity of infection (Fig. 8). Hence, transactivation could be reached by a very strong overexpression of p53, a result that better fits the model of a dominant inhibitor acting in RCC cells.

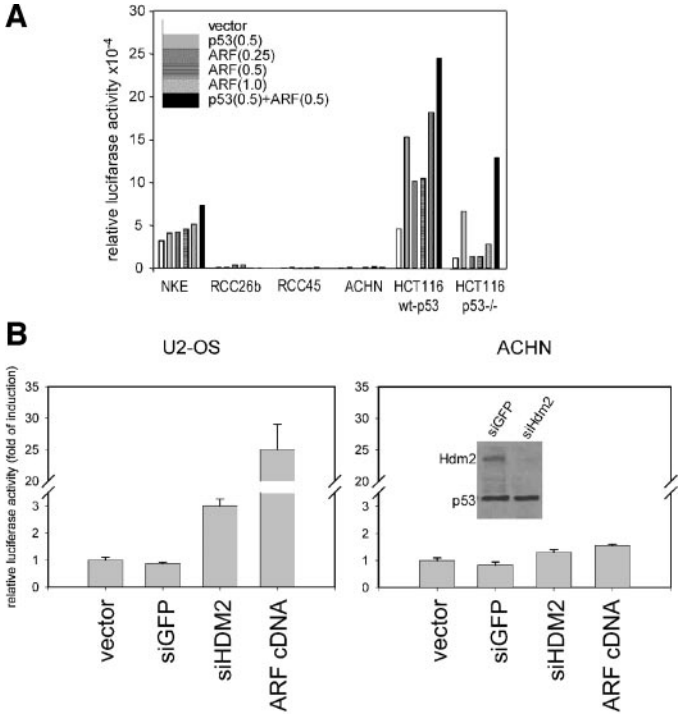

Fig. 7. Functional testing of Arf and Mdm2 involvement in the p53 transactivation defect in RCC cells. *A*, influence of reconstituted ARF expression on p53-responsive reporter activity. Cells in 6-well plates were transfected with p21-ConA-Luc (0.5 μg) and the indicated amounts of pLXSP, pLwtp53SP, or pcDNA3ARF. pCMVLacZ vector (0.1 μg/well) was added in all mixtures to estimate transfection efficiency by measuring β-galactosidase activity. Data represent the ratio of luciferase activity normalized by transfection efficiency. *B*, ectopic expression of Arf and knockdown of Mdm2 enhance the activity of p53 luciferase reporter in U2-OS cells, but not in ACHN cells. U2-OS and ACHN cells carrying integrated p21-ConA-Luc reporter were transduced with amphotropic retroviruses expressing the indicated constructs, and luciferase activity was measured after 3 days of cell selection in the presence of 1 μg/ml puromycin. *Bars* represent relative luciferase activity normalized by cellular protein in the lysates. The insert demonstrates Hdm2 and p53 protein levels in ACHN cells transduced with the indicated constructs.

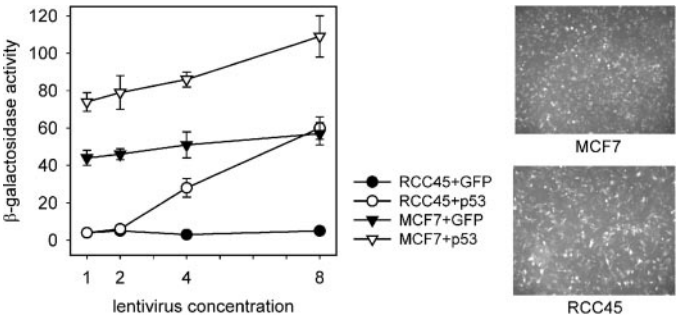

Fig. 8. p53-responsive reporter activity in cells transduced with lentiviral vectors bearing green fluorescent protein (GFP) or p53 cDNA. MCF7ConALacZ or RCC45ConALacZ cells in 96-well plates were transduced with different dilutions of either GFP or p53 lentiviral stocks. Lentivirus concentrations used for infection (indicated as transduction units $\times 10^{-7}$) are indicated. Reporter activity was measured 48 h later in lysates of cells. Data represent β-galactosidase activity normalized by protein concentrations. Microphotographs of cell monolayers transduced with the same dilutions of GFP lentivirus are shown to illustrate the comparable transduction efficiency of cells.

This possibility received additional support with the results of experiments involving polyethylene glycol-mediated cell fusion to create heterokaryons of RCC cells with p53-deficient H1299 cells carrying an integrated p53-responsive reporter construct (indicator cells) followed by *in situ* (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining) analysis of p53 transactivation function 24 h after fusion. H1299 cells preserve the intact p53 pathway and activate the reporter in response to ectopic expression of p53 (Fig. 1*C*). MCF7 cells characterized by active endogenous p53 served as a positive control in cell fusion experiments, causing pronounced activation of expression of the reporter in heterokaryons. HeLa cells served as an example of cells with p53 suppressed by a dominant mechanism (viral E6 protein); they were incapable of activating the reporter in H1299 after fusion.

None of the four studied RCC cell variants activated the p53-responsive reporter in the H1299 cells after fusion (Fig. 9, Table 1). Moreover, fusion with the RCC cells inhibited reporter activity in MCF7 as compared with the MCF7 cells fused with themselves or with H1299 cells. The result was the same regardless of which fusion partner (RCCs, MCF7, or H1299) carried the reporter. No reporter activity was detected in heterokarions of RCC45 cells carrying p21-ConA-LacZ with any of the remaining RCC cell lines studied. Taken together, these observations indicate that p53 transactivation is repressed in RCC cells by a dominant mechanism.

NKE cells activated reporter activity after fusion with indicator H1299 cells to a significantly lesser extent than MCF7 cells (Fig. 9, Table 1). NKE cells also reduced reporter activation in indicator MCF7 cells in comparison with MCF7 cells fused with themselves (Fig. 9). Finally, fusion of NKE cells with RCC45 cells carrying p21-ConA-LacZ reporter did not reactivate reporter expression. Hence, NKE seems to possess weak p53 suppressive activity, suggesting that the putative dominant inhibitor of p53-dependent transactivation in RCC cells is tissue specific and active in normal kidney cells.

DISCUSSION

The high frequency of *p53* mutations in cancer reflects the importance of p53 inactivation for tumorigenic processes. However, the

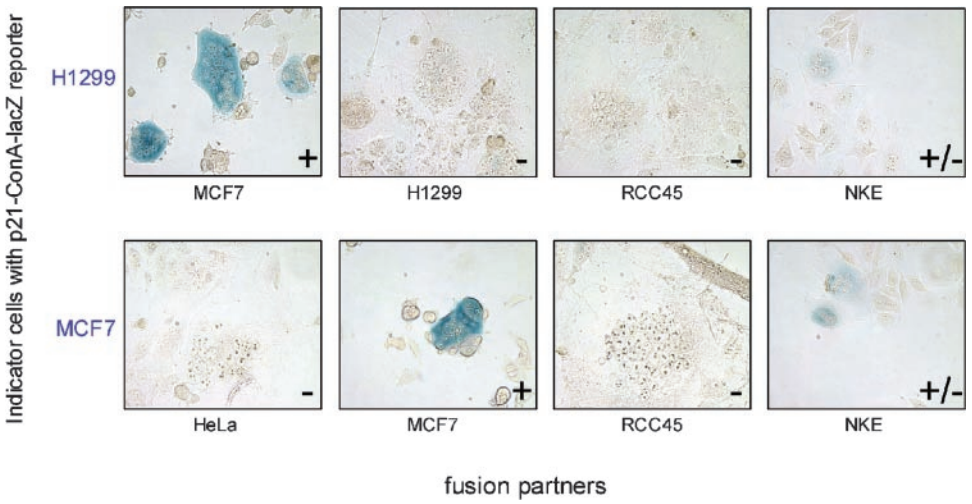

Fig. 9. Fusion of renal cell carcinoma cells and cells with known p53 status. Microphotographs of polynucleated cells resulting from polyethylene glycol-mediated fusion of the indicated partners and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for β-galactosidase expression 24 h after fusion (see "Materials and Methods") are shown.

frequency of p53-inactivating mutations depends greatly on the type of cancer, presumably indicating differences in p53 function and regulation in different tissues. For example, point mutations within the p53 gene were observed in 60–65% of lung and colon cancers; 40–45% of stomach, esophagus, and bladder cancers; 25–30% of breast, liver, and prostate cancers and lymphomas; and in only 10–15% of leukemias (6, 7). In some types of cancer, such as cervical carcinomas, melanomas, or RCC, p53 mutations are even less frequent. Importantly, a close look at those tumors that maintain expression of wild-type p53 indicates that in the majority of them, p53 function is also inactivated. In some cases, the mechanisms of such inactivation are known: p53 signaling can be repressed by deregulation of p53-regulatory proteins (such as Arf, Mdm2, or MdmX) or expression of viral oncogenes, inhibitors of p53. The latter mechanism is responsible for p53 inactivation and lack of p53 mutations in cervical carcinomas, the vast majority of which originate from cells permanently infected by human papilloma virus that express E6 protein, promoting p53 degradation. All of the above suggest that p53 pathway repression is a prerequisite for tumor development and predicts that those tumors that maintain wild-type p53 almost certainly acquire some other mechanisms of its functional repression. Identification of these mechanisms is important for understanding the specific genetic basis for various types of cancer and is likely to result in defining new targets for anticancer treatment.

RCC belongs to the type of tumors with low incidence of p53 mutations (6–12). However, p53 studies in RCC have been mostly limited to the analysis of tumors; neither the functionality of p53 signaling nor mechanisms of p53 inactivation in RCC have ever been addressed. We analyzed this problem using a set of RCC-derived strains and cell lines and studied them side by side with p53 wild-type tumor cell lines of different origin in which the status of the p53 pathway has been well determined and with normal kidney epithelia growing in culture under similar conditions. Although our study was limited to four RCC strains, we were able to make general conclusions because the results obtained appeared to be very consistent within the group. In fact, all RCC variants studied were found to express wild-type p53, which, in all variants, was deficient in its transactivation function. Similar situations have been described previously in other tumor types, in which p53 function was repressed by overexpression of its natural negative regulators, Mdm2 and MdmX (13–15) or viral oncoproteins [E6 (18)], by cytoplasmic sequestration of p53 through binding with anchoring protein [Parc (32)], or by loss of Arf, an important p53 cooperator encoded by a tumor suppressor gene (17, 30). We therefore started analysis of RCC by testing all these anticipated options and had to rule them all out because, in response to DNA damage, p53 becomes stabilized, translocates and accumulates in the nuclei, and becomes capable of specific DNA binding. Lack of Arf expression in some of the RCC cells was found to be irrelevant to the observed suppression of p53 function, as became clear from the lack of p53-activating effect of ectopic reconstitution of Arf expression.

Hence, in RCC cells, p53 undergoes all expected conversions in response to DNA damage but paradoxically does not induce transactivation. This situation could be explained either by lack of some p53 counterpart that is essential for transactivation or by the presence of an inhibitor of transactivation. To discriminate between the two possibilities, we carried out a series of cell fusion experiments with monitoring of p53 transactivation function in cell-cell hybrids, and the obtained results unequivocally pointed to a dominant mechanism of p53 repression.

Discrimination between the two scenarios is important for further identification of the exact mechanism of p53 dysfunction in RCC and for choosing a strategy to develop a p53 pathway-targeted approach to anti-RCC treatment. We presume that reactivation of p53 function in RCC could either cause a direct killing of tumor cells or sensitize them to anticancer treatment. If p53 were inactive because of a missing counterpart (recessive mechanism), then its activity could be restored by reconstitution of a missing factor, a task that would require gene therapy and therefore would have questionable clinical perspectives. However, because p53 in RCC is repressed by a dominant factor, this creates a "druggable" situation by opening the opportunity of generating molecules interfering with the inhibitory factor.

Table 1 *Summary of fusion experiments*

Cells used for fusion	H1299	MCF7	HeLa	RCC26	RCC45	RCC54	ACHN	NKE
H1299-ConA-LacZ	−[a]	+	−	−	−	−	−	+/−
MCF7-ConA-LacZ	+	+	−	nd	−	nd	nd	+/−
RCC45-ConA-LacZ	−	−	nd	−	−	−	−	−

[a] −, complete absence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside positive heterokarions; +/−, weakly stained heterokarions; +, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside positive heterokarions; nd, not done.

The fact that there is a detectable repression of p53 transactivation in normal kidney cells that is also dominant in cell fusion experiments allows us to hypothesize that RCC cells may use an existing kidney-specific mechanism of p53 attenuation to achieve complete inhibition of the p53 pathway. Identification of the RCC-specific p53-inhibitory factor is the next step of this work that we will begin by testing obvious "suspects": p53-interfering proteins encoded by the genes belonging to the p53 superfamily (p63 and p73). Whatever the nature of this p53 inhibitor, we hope that it will be an "Achilles heel" of RCC that can be used for development of a new effective treatment.

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